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Morphological and morphometric changes in the gills of mosquitofish (*Gambusia holbrooki*) after exposure to mercury (II)

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Abstract

Physiological measurements suggest that mercury (Hg) affects ion regulation in aquatic organisms. This implies that Hg should cause morphological changes in gills, the major ionoregulatory organ of fish. Previous studies have shown severe Hg-induced gill damage at acutely lethal Hg concentrations, but in soft fresh waters, where ionoregulatory disturbances should be most pronounced, there have been no quantitative studies to date of the effects of dissolved Hg at sublethal concentrations. We exposed mosquitofish (*Gambusia holbrooki*) to nominal concentrations of 75, 150 and 300 nM Hg (II) in natural stream water of low ionic strength (Ca = 4.8 μM) in a static-renewal experiment. Mercury concentrations dropped rapidly after Hg additions, and most dissolved Hg was probably complexed with humic substances. Gills were sampled after 7 and 14 days exposure. Examination with scanning electron microscopy (SEM), indicated that Hg caused progressive loss of secondary lamellae and loss of cell surface microridges. Effects became more severe at higher Hg concentrations. Using light microscopy and morphometric techniques, the percent of gill filament occupied by chloride cells (volume density) was significantly greater in all Hg treatments than in controls, and thickness of the primary lamellar epithelium increased with Hg exposure. Thickening of the primary lamellar epithelium filled some interlamellar spaces, obliterating secondary lamellae. However, width of the exposed portions of secondary lamellae was not affected by Hg, suggesting that blood-to-water diffusion distances did not increase in response to Hg exposure. Autometallographic staining for Hg showed that the metal was distributed throughout the gill epithelium, and not concentrated inside chloride cells.

Keywords: Mercury; Chloride cells; Gill epithelium; SEM; Stereology; Autometallography

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1. Introduction

In fish, gills are the main site of osmotic water movement and ion flux, and are therefore crucial to processes of ion- and osmoregulation. Gills are also the major organ responsible for acid–base regulation and nitrogenous waste (ammonia) excretion, as well as respiratory gas exchange. A variety of pollutants interfere with branchial fluxes of gases and electrolytes (Wood, 1992), including low environmental pH (McDonald, 1983), Al (Booth et al., 1988), Cu (Lauren and McDonald, 1985), Cd (Larsson et al., 1981), and Zn (Spry and Wood, 1985). Available evidence indicates that Hg also affects ionic homeostasis. Waterborne Hg inhibits the activity of gill Na^+,K^+-ATPase (Renfro et al,. 1974; Borquegneau, 1977), an enzyme responsible for active ion uptake by the gill epithelium. Lock et al. (1981) showed that dissolved Hg altered the permeability characteristics of gills, increasing passive ionic effluxes. Additionally, Hg accumulated via the diet, a more environmentally realistic route of uptake (Phillips and Buhler, 1978; Dallinger et al., 1987), also seems to disrupt ion regulation in fish and other organisms (Stagg et al., 1992; Wright and Welbourn, 1993).

Because gills are the major organ involved in ion regulation and related activities in fish, alterations in gill morphology typically accompany alterations in these physiological processes (Laurent et al., 1985; Evans et al., 1988; Tietge et al., 1988; Perry et al., 1992). Morphological changes in gills may cause altered fluxes, or represent a response to such alterations. As an example of the former case, changes in the structure of intercellular junctions upon exposure to low pH increase gill permeability, in turn increasing ionic effluxes (Freda et al., 1991). As an example of the latter, an increase in the number of ion-transporting cells may be viewed as a compensatory response to offset ionic losses upon low pH exposure (Leino and McCormick, 1984; Jagoe and Haines, 1990). Regardless of whether morphological changes are viewed as cause or effect, alterations in structure and function are highly correlated in gills.

Given that Hg affects gill processes, it might be expected to affect gill structure as well. Several studies have documented severe gill damage upon exposure to extremely high concentrations of inorganic and organic forms of Hg (Wobeser, 1975a; Khangarot and Somani, 1980; Daoust and Wobeser, 1984; Paulose, 1987), and this damage is often associated with significantly increased mortality. Few studies have examined longer-term effects of exposure to sublethal concentrations of Hg on gill morphology, although one recent study (Pereira, 1988) documented the responses of gills of flounder (*Scophthalmus aquosus*) exposed to 25–50 nM Hg in seawater for 60 days. To our knowledge, no studies to date have examined the responses of gills to sublethal levels of dissolved Hg in freshwaters of low ionic strength, where the effects of metals on ionoregulatory processes are most pronounced (Lauren and McDonald, 1985; Spry and Wood, 1985; McDonald et al.,1989). Also, almost all studies of Hg-induced alterations in gill morphology to date have been purely qualitative, without consideration of quantitative changes in parameters such as diffusion distance or number and size of ion-transporting cells. Quantitative studies of morphology are often essential to detect subacute effects, and to facilitate comparison of responses at the cell and tissue levels with effects at other levels of organization (Bolender, 1979; Hinton et al.,

1987; Hinton, 1993; Jagoe, 1995). To address these considerations, we selected mosquitofish, *Gambusia holbrooki*, as a model species, and exposed them to sublethal doses of inorganic Hg at three concentrations in a natural soft water. We then used scanning electron microscopy and quantitative light microscopy to evaluate the effects of Hg exposure on gill morphology, with particular emphasis on those features associated with ion regulation.

2. Materials and methods

Adult mosquitofish (total length 12–30 mm) were collected from Risher Pond on the US Department of Energy's Savannah River Site (SRS), near Aiken SC in June 1991. The pond had no known history of pollution, and was located in a forested catchment with no residential, agricultural or industrial activities in the watershed. Fish were collected using seines and dip nets, then returned to the laboratory for acclimation to the test water for 3 weeks before the initiation of Hg exposures. During the acclimation period, fish were held in a large fiberglass tank and fed a commercial flake diet (Tetra-Min®) and frozen brine shrimp daily. The water was continuously aerated, and temperature was maintained at 20–23°C.

Fish were acclimated to water collected from Upper Three Runs Creek on the SRS, and all experimental exposures were conducted in this water. The watershed of the creek is largely undeveloped, although the headwaters drain some agricultural areas. Previous studies indicated that the water was soft (specific conductance 15–30 μmhos/cm), near neutral in pH and did not contain significant amounts of potentially toxic trace metals (Newman, 1986). Water was collected at the stream into a 500-l plastic tank about once per week, and transported to the laboratory for use.

After 3 weeks of acclimation, groups of 20 fish were transferred to one of 12 polyethylene tanks, each containing 6 l of water. Each aquarium was randomly assigned a treatment of (nominally) 0, 75, 150 or 300 nM Hg, yielding 3 replicates per treatment. Each was continuously aerated through a glass tube. Water in each aquarium was replaced daily with fresh water spiked (from a 50 μM stock solution of $HgCl_2$) with the appropriate amount of Hg. Temperature was recorded daily for each tank, and ranged from 21 to 26°C. Water samples were collected daily for determination of pH. A subset of these samples was also analyzed for acid neutralizing capacity (ANC), ammonia, selected cations and dissolved organic carbon (DOC). Ammonia was measured by ion-specific electrode, and ANC by double endpoint titration (American Public Health Association et al., 1985). Na, K and Ca were measured by flame atomic absorption spectrophotometry, and DOC was determined by high-temperature catalytic oxidation with infrared CO_2 detection.

An experiment was done to determine the stability of Hg concentrations in the experimental aquaria before fish exposures began. Three aquaria were filled with stream water, and Hg (nominally 75, 150 or 300 nM) was added. Water samples were collected from just beneath the water surface into acid-cleaned glass bottles immediately after the additions, and after 4, 8, 12 and 24 h. A control aquarium (stream water with no Hg added) was sampled simultaneously and the experiment was repeat-

ed after the fish exposures. Water samples were collected in the same manner during the fish exposures, and in the first week of exposure, water for Hg analysis was collected twice per day. One sample was collected within 1 h of the water change, and another just before the water was changed again (24 h). During the second week of the exposures, water samples were collected for Hg analysis every other day, within 1 h after the water was changed. For determination of total Hg, water samples were digested with HNO_3, H_2SO_4, $KMnO_4$, and $K_2S_2O_8$ in closed bottles in a water bath at 95°C for 2 h (American Public Health Association et al., 1985). Digested water samples were analyzed for Hg using cold-vapor atomic absorption spectroscopy.

Half of the fish in each treatment were removed and fixed after 7 days exposure, and the remainder at the end of 14 days. Whole fish were fixed in 0.1 M HEPES buffer (Sigma Chemical), pH 7.4, containing 1% (v/v) glutaraldehyde and 4% (w/v) formaldehyde (from paraformaldehyde; Electron Microscopy Sciences). Fish were stored in fixative under refrigeration until prepared for light or electron microscopy.

The second and third gill arches from both left and right sides were carefully removed from individual fish and placed in containers made from BEEM capsules (Electron Microscopy Sciences) and plankton netting. These containers allow free exchange of solutions; by arranging them in a petri dish, it was possible to change solutions without disturbing the individual gill arches, and to assure that all tissues were exposed to the same dehydration regime. Tissues were dehydrated through a progressive ethanol series from 50 to 100% ethanol.

Gills from fish collected after 7 and 14 days exposure were examined by scanning electron microscopy (SEM). For SEM, the dehydrated gills arches were critical-point dried using CO_2, mounted on aluminum stubs and sputter-coated with gold. Gills were examined using an AMR 1000 scanning electron microscope equipped with a LaB_6 cathode operated at 30 kV, and photographed on Polaroid Type NP-55 film.

Only gills from fish exposed to the experimental conditions for 14 days were used for light microscopy and quantitative histopathology. Gill arches for light microscopic examination were transferred from ethanol to propylene oxide, then embedded in an Epon-Araldite mixture. The embedded gills were sectioned to a nominal thickness of 1 μm and mounted on slides. The plane of sectioning was parallel to the gill arch and the primary lamellae, and perpendicular to the secondary lamellae. Each section included a number of primary lamellae cut longitudinally. Slides for quantitative measurements were stained with 1% (w/v) toluidine blue. Some slides were stained using periodic acid-Schiffs reagent (PAS; Humason, 1967) to visualize mucous cells. To visualize mercury within tissues, additional slides were stained using an autometallographic procedure based on the catalytic transformation of silver ions from solution to metallic silver by mercury bound to sulfur or selenium in tissue (Danscher, 1984; Jensen and Baatrup, 1988; Andersen and Baatrup, 1988).

For quantitative measurements, gills were examined using a 40× objective on a Leica Dialux 22 microscope equipped with a color video camera and monitor. Final magnification on the monitor screen was measured at 1800× with a stage micrometer. Two individuals from each aquarium (six individuals per treatment) were randomly selected for morphometric analysis, and two slides, prepared from slightly different regions of the gill, were examined per individual. All slides were coded and

examined in random order, so the individual examining the slides did not know the treatment associated with any slide. The volume density of chloride cells was measured by a stereological technique (point counting; Weibel et al., 1966; Elias, 1983). A 144-point square grid was placed on the monitor screen, with sections randomly oriented with respect to the grid. A total of five fields per slide (ten per individual) were examined, each consisting of a portion of a primary lamella and several secondary lamellae. The number of points in each field falling in lamellar tissue and in chloride cells were recorded, and the volume density of chloride cells calculated as a percentage of the total lamellar tissue in the field.

Epithelial thickness is not uniform, and it is necessary to avoid bias towards areas of maximum or minimum thickness to accurately compare mean thickness among individuals. For determination of thickness of the primary lamellar epithelium, we used an 81-point Merz grid, randomly oriented with respect to the section, and measured distances from the point where a test line intersected the outer epithelial surface to the basal lamina. This method randomizes the location of the measurement (Hughes and Perry, 1976; Hughes et al., 1979) and has been widely applied to the measurement of gill epithelial thickness (Tietge et al., 1988; Leino and McCormick, 1993; Wilson et al., 1994). Measurements were made on the monitor screen to the nearest mm using a transparent ruler. Sites were selected in the same random manner to determine the width of individual secondary lamellae, and the distance from the intersection of the test lines with the outer edge of the epithelium to the nearest point on the epithelial surface on the opposite side of the secondary lamella was measured. Five measurements each for primary lamellar epithelial thickness and secondary lamellar width were made from each slide, resulting in a total of ten measurements each per individual.

Stereological and morphometric measurements were analyzed by ANOVA (SAS-PC version 6.04; SAS Institute, Cary, NC). The chloride cell volume density values are proportions, so these were arcsin-square root transformed before analysis (Sokal and Rohlf, 1981). The variances of the epithelial width measurements were similar among the treatments, and the distribution of these measurements did not depart significantly from normality (by Komolgorov-Smirnov test), so these were not transformed. Treatments that differed significantly ($P < 0.05$) from the control values were identified using Dunnetts' t test.

3. Results

Water chemistry

Over the 2-week fish exposure period, pH in the aquaria averaged 6.79 (standard deviation 0.29). Ammonia concentrations never exceeded 24 μM, and were typically < 0.10 μM. Mean Na concentration in the water ($\pm$ standard error) was 8.2 ± 1.8 μM ($n = 36$ measurements). Mean Ca concentration was 4.8 ± 0.1 μM, and mean K concentration 0.50 ± 0.04 μM ($n = 36$ each). Mean DOC concentration was 4.7 ± 0.3 mg/l, and mean ANC was 148 ± 11 μeq/l ($n = 36$ each). None of these water chemistry parameters varied significantly among the treatments. We did not measure Mg, but

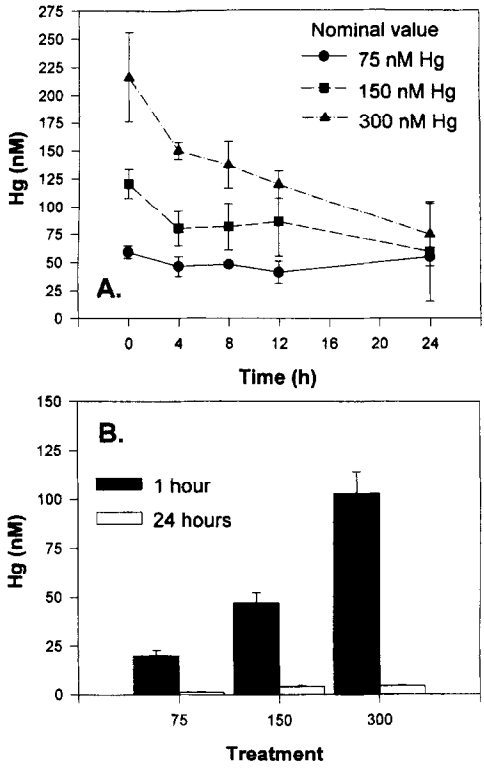

Fig. 1. (A) Change in Hg concentration in 75, 150, and 300 nM (nominal) treatments (without fish) over 24 h. Mean Hg concentration ± standard error, $n = 2$ determinations per data point, from 2 replicate experiments. (B) Change in Hg concentrations in 75, 150, and 300 nM (nominal) treatments during fish exposures. Mean Hg concentration ± standard error, $n = 30$ determinations per treatment per time.

in a previous study, the average Mg concentration of Upper Three Runs water was 13.7 μM (Newman, 1986).

In the experiments to determine the stability of Hg concentrations in the aquaria without fish present, the initial (time = 0) Hg concentrations were close to the nominal values (Fig. 1A). Over 24 h, Hg concentration in the 75 nM treatment was relatively constant, while Hg concentrations in the 150 nM and 300 nM treatments declined (Fig. 1A). At the end of 24 h, Hg concentrations in all three treatments were essentially identical.

Hg concentrations were below detection in all samples collected from the control treatments. As was the case when fish were not present, water Hg concentration decreased in the experimental treatments when fish were present, although the decrease over time was much more pronounced. Analyses of samples taken during the fish exposures showed that within 1 h of the addition of fresh, Hg-spiked test water, the Hg concentration in each experimental treatment was approximately 1/3 of the nominal value (Fig. 1B). After 24 h (just before the next water change), Hg concentra-

tions in all of the treatments had decreased to less than 5 nM (Fig. 1B). Thus, fish in the experimental treatments experienced pulsed exposures, with a fresh pulse of Hg-enriched water every 24 h, and periods of decreasing Hg concentration between pulses.

It is important to note that, although Hg concentrations declined rapidly over time during the fish exposures, the magnitude of differences in Hg concentrations among the treatments measured soon after water renewal were the same as those expected for the nominal concentrations. That is, the measured Hg concentration in the nominally 300 nM treatment was twice that measured in the 150 nM treatment, and the concentration in the 150 nM treatment was twice that in the 75 nM treatment. Based on these measurements, it is reasonable to conclude that fish in the highest Hg treatment experienced twice the Hg exposure of fish in the intermediate Hg treatment, and that fish in the intermediate treatment were exposed to twice as much Hg as fish in the low treatment during the pulse. For simplicity, we refer to the treatments by their nominal Hg concentrations when considering effects on gill structure. However, we emphasize that the nominal concentrations reflect only initial conditions, and concentrations declined very rapidly between pulses of Hg-enriched water.

Mortality

Some fish died during the experiment, but this mortality was not directly related to Hg concentration. One control fish died during the 14 days of exposure, 3 fish died in both the 75 nM and 300 nM treatments, and 4 fish died in the 150 nM aquaria. Deaths occurred at irregular intervals during the exposure, and did not appear to increase in frequency with time. We conclude that the Hg concentrations used were not acutely toxic, but probably were sufficient to cause physiological stress.

Surface morphology

After 14 days exposure to the experimental treatments, gills of control fish appeared normal and well formed (Fig. 2A). Two rows of filaments or primary lamellae projected from the gill arch, and each primary lamella had two parallel rows of respiratory lamellae or secondary lamellae. No fusions between adjacent secondary lamellae were observed. Primary and secondary lamellae of fish from the 75 nM Hg treatment appeared swollen or thickened (Fig. 2B). Some secondary lamellae appeared to be joined together, especially near the bases of the primary lamellae. In fish from the 150 nM treatment, the swelling or thickening of the primary lamellae was more pronounced (Fig. 2C). Regions where secondary lamellae were absent appeared more numerous, and the height of individual secondary lamellae, from their origin at the primary lamella to their tips, appeared shortened. Gills of fish from the 300 nM treatment had grossly thickened primary lamellae, and large areas appeared devoid of secondary lamellae, because adjacent lamellae had joined into a continuous surface (Fig. 2D). Regions where adjacent primary lamellae were partially to completely joined were also present in fish exposed to this treatment.

Examination of gills from fish collected after 7 days exposure suggested that changes in gill morphology had begun by 7 days, but became more severe by 14 days. In the 150 nM treatment, fish had some shortened secondary lamellae, and some regions where secondary lamellae were not visible after 7 days of exposure (Fig. 3A). How-

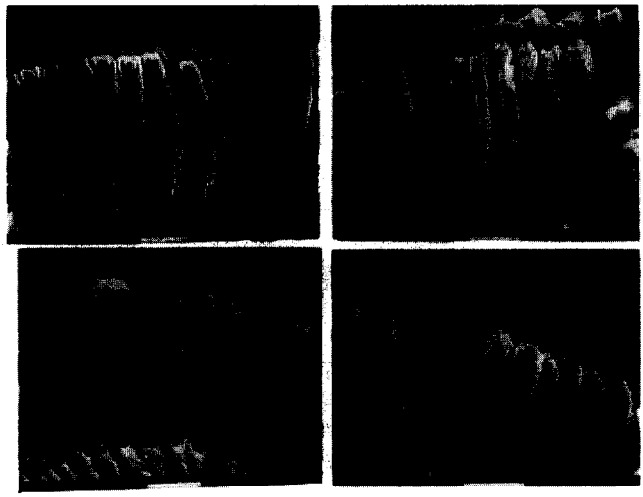

Fig. 2. Scanning electron micrographs of gills of mosquitofish after 14 days in experimental treatments: (a) control, (b) 75 nM Hg, (c) 150 nM Hg, (d) 300 nM Hg. Scale bars = 100 μm.

ever, primary lamellae did not appear as swollen or thickened as those exposed for 14 days (compare to Fig. 2C). Fish in the 300 nM treatment also had regions where individual secondary lamellae were not visible after 7 days exposure (Fig. 3B); these regions appeared more extensive than those in fish held in the 150 nM treatment for the same period. The regions where secondary lamellae were shortened or absent were not as extensive as those observed after 14 days in the 300 nM treatment, and no fusions of adjacent primary lamellae were seen after 7 days of exposure. These observations support the conclusion that swelling or thickening of the primary lamellae with progressive shortening or loss of secondary lamellae occurs with increasing Hg concentration and length of exposure.

Changes in the surface ultrastructure of epithelial cells associated with Hg exposure were visible at higher magnification. The pavement cells of fish from control treatments, especially those on the primary lamellar surfaces, were covered with abundant microridges resembling fingerprints (Fig. 4A). After 14 days exposure to the 75 nM treatment, epithelial surfaces appeared more uneven and there was a noticeable de-

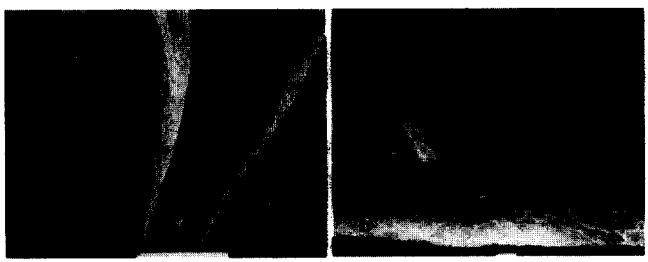

Fig. 3. Scanning electron micrographs of gills of mosquitofish after 7 days exposure to experimental treatments: (a) 150 nM Hg, scale bar = 100 μm, (b) 300 nM Hg, scale bar = 10 μm.

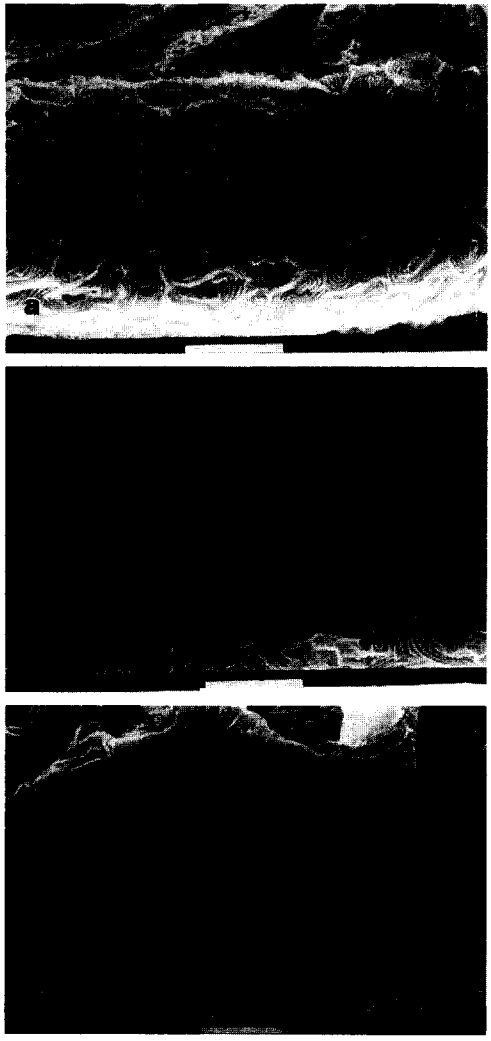

Fig. 4. Scanning electron micrographs of surfaces of primary lamellar epithelia of mosquitofish after 14 days exposure to experimental treatments: (a) control, (b) 75 nM Hg, (c) 300 nM Hg. Scale bars = 10 μm.

crease in the height and density of the microridges (Fig. 4B). Chloride cells were distinguishable by their slightly recessed apical surfaces, and their number appeared higher than in the control treatments. After 14 days exposure to the 300 nM treatment, microridges were absent over large portions of the epithelial surface (Fig. 4C). Chloride cells also appeared more numerous, with larger apical surfaces, than in lower Hg treatments.

Internal morphology

Sectioned gills from control fish examined using light microscopy had distinct,

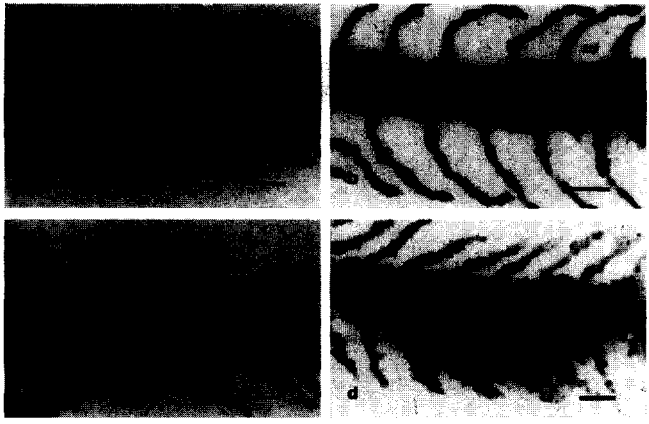

Fig. 5. Micrographs of sectioned gills of mosquitofish after 14 days exposure to experimental treatments stained with toluidine blue: (a) control; arrows indicate representative chloride cells, distinguishable by lightly stained cytoplasm, (b) 75 nM, (c) 150 nM Hg, (d) 300 nM Hg. Scale bars = 20 μm.

regular secondary lamellae (Fig. 5A). The primary lamellar epithelium was one or two cell layers thick. Chloride cells (distinguishable by their lightly-stained cytoplasm, Fig. 5) were visible along the primary lamellar epithelium, especially at the bases of secondary lamellae. Chloride cells were also occasionally observed on secondary lamellae but this was uncommon. In material stained by the PAS technique, mucous cells appeared sparsely scattered along the primary lamellar epithelium and less frequently along the secondary lamellae.

The structure of the primary lamellae was visibly altered in fish exposed to the Hg treatments. In the 75 nM treatment, chloride cells appeared larger and more numerous than in controls (Fig. 5B). The primary lamellar epithelium appeared thickened in gills from fish exposed to this treatment. This thickening seemed regular, without noticeably thicker or thinner regions along the primary lamellae. Fish from the 150 nM treatment also had increased numbers of chloride cells in their gills. In many interlamellar spaces, chloride cells occupied the entire primary lamellar surface (Fig. 5C). As in the 75 nM treatment, the primary lamellar epithelium appeared thickened, but in the 150 nM treatment the thickening was noticeably irregular. In fish from the 300 nM treatment, the thickening of the primary lamellar epithelium was more pronounced, and highly irregular (Fig. 5D). Numerous chloride cells were present, often in clusters or multiple layers.

Secondary lamellar epithelia were less affected by Hg exposure. Chloride cells did not proliferate along the secondary lamellae in Hg-exposed fish. The thickness or width of secondary lamellae also appeared relatively constant in control or Hg-exposed fish (Figs. 5A–D). We did not observe areas of epithelial detachment or edema associated with Hg exposure. However, secondary lamellae in Hg-exposed fish often appeared shorter than in control fish, because the increased thickness of the primary lamellar epithelium filled the interlamellar spaces (Figs. 5C, 5D).

We did not quantitatively examine changes in mucous cell size or number. Based on

qualitative observations of a few slides from each treatment stained to demonstrate mucous cells, the number of mucous cells did not appear to increase with Hg exposure.

Fish sampled after 14 days exposure were used to examine Hg distribution within gill tissues. There was insufficient material available from the 75 nM treatment, so only gills from fish in the 0, 150 and 300 nM treatments were examined. In fish exposed to Hg in the 150 and 300 nM treatments, autometallographic staining showed granular deposits within the gill epithelium (Fig. 6A, 6B). These particles were not observed in slides from control fish stained in the same manner. At lower magnification, the particles appeared to be distributed throughout the primary lamellar epithelium, and to a lesser extent, the secondary lamellar epithelium (Fig. 6A). Examination at higher magnification did not show the particles associated with a particular cell type (Fig. 6B). The granules were more abundant in the outer cell layers of the epithelium, but were sometimes present in deeper portions as well. Although chloride cells were difficult to identify without additional staining, the larger and more lucent cells located in positions where chloride cells might be expected contained relatively few particles. These results suggest that Hg was not accumulated in chloride cells under the experimental conditions.

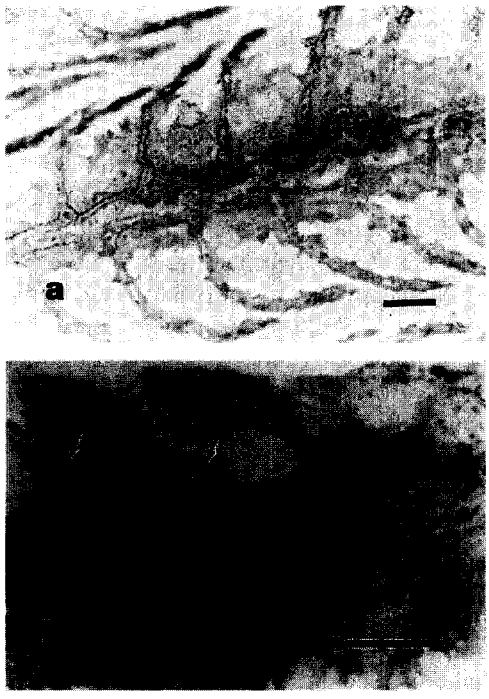

Fig. 6. Micrographs of sectioned gills of mosquitofish after 14 days exposure to experimental treatments stained for Hg by Danscher's autometallographic method: (a) 300 nM Hg, (b) 300 nM Hg; arrows indicate some probable chloride cells. Scale bars = 20 μm.

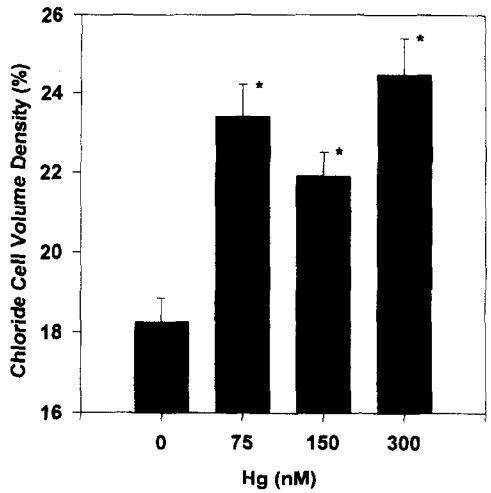

Fig. 7. Chloride cell volume density after 14 days exposure to the experimental treatments. Mean volume density ± standard error, $n = 60$ measurements per treatment. Asterisks indicate significant differences from control value, $P < 0.05$.

Stereology and morphometry

The volume density of chloride cells significantly increased with Hg exposure, as measured by the stereological technique of differential point counting. In control fish, chloride cells accounted for about 18% of the tissue volume in the lamellae. The volume density increased to 22–25% in Hg-exposed fish (Fig. 7). Chloride cell volume density was higher in all experimental treatments than in controls, and fish exposed to the 300 nM treatment had the highest chloride cell volume density.

Mean thickness of primary lamellar epithelia tended to increase with Hg exposure (Fig. 8A). In fish from the 300 nM treatment, mean epithelial thickness on primary lamellae was significantly greater than in control fish, but in fish from the 75 nM and 150 nM treatments, thickness of the primary lamellar epithelium was not significantly different from the control value. There was no apparent trend in thickness of secondary lamellae with Hg concentration (Fig. 8B). In the three Hg treatments, secondary lamellar thickness did not differ significantly from the control value.

4. Discussion

In fish exposed to sublethal concentrations of dissolved Hg (II) in our experiment, the major responses in gills were an increase in the volume density of chloride cells, thickening of the primary lamellar epithelium, and changes in the fine structure of the surfaces of the epithelial cells. The thickening of the primary lamellar epithelium filled the space between secondary lamellae in some instances, so secondary lamellae appeared to shorten and disappear. We did not observe extensive necrosis, detachment of the secondary lamellar epithelium, or extensive edema, as have been reported upon

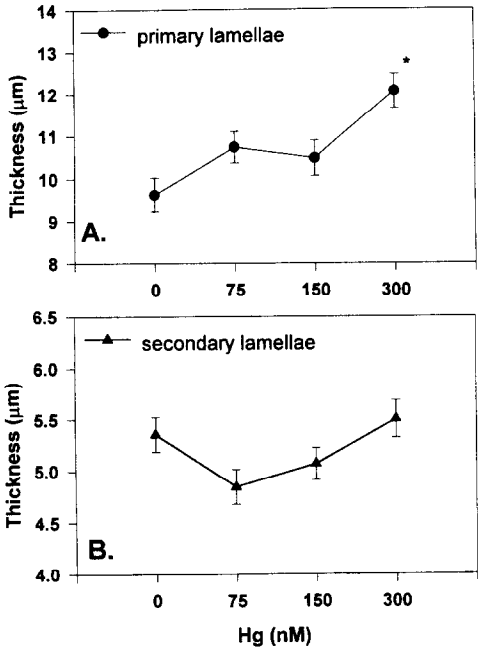

Fig. 8. (A) Thickness of primary lamellar epithelium after 14 days exposure to the experimental treatments. Mean thickness ± standard error, $n = 60$ measurements per treatment. (B) Thickness or width of secondary lamellae after 14 days exposure to the experimental treatments. Mean thickness ± standard error, $n = 60$ measurements per treatment. Asterisks indicate significant differences from control value, $P < 0.05$.

exposure to acutely lethal concentrations of Hg (Wobeser, 1975a; Khangarot and Somani, 1980; Daoust and Wobeser, 1984; Paulose, 1987). Very high concentrations of Hg apparently cause gross tissue damage, but such extensive pathological responses do not occur at lower doses.

The shortening and progressive disappearance of secondary lamellae that we observed are similar to the responses produced by other metals, including Al (Karlsson-Norrgren et al., 1986), Be (Jagoe et al., 1993) and Cd (Karlsson-Norrgren et al., 1985). Many of the cells in the interlamellar spaces were morphologically recognizable as chloride cells, the site of active ion uptake in gills of freshwater fish (Laurent et al., 1992; Perry et al., 1992). The abnormal gill morphology we observed is consistent with altered ion flux at the gills, as previously reported by Lock et al. (1981). Ionic homeostasis is affected by a number of dissolved metals (Larsson et al., 1981; Lauren and McDonald, 1985; Spry and Wood, 1985; Booth et al., 1988), and our results support the conclusion that Hg (II) elicits a similar response.

The bioavailability and toxicity of dissolved metals vary with speciation, and therefore consideration of Hg (II) speciation under our experimental conditions is essential for interpretation of our findings. Speciation depends on the activities of inorganic and organic ligands that may complex a particular metal. We used water collected from a natural stream, containing dissolved organic materials (average DOC = 4.7

mg/l). Organic ligands readily complex Hg, although stability constants are generally available only for low molecular weight compounds. The scarcity of empirical data concerning binding of Hg to high molecular weight humic substances makes prediction of Hg speciation difficult in DOC-rich waters. Benes and Havlik (1979) surveyed a number of studies and reported estimates of the percentage of total Hg complexed to humic substances in natural waters ranging from 25 to over 95%. In soft water where fewer competing inorganic ligands are present, it seems likely that more added Hg would complex with dissolved organic matter. Also, the concentration of dissolved organic matter in the water we used was much greater than the concentration of Hg added. Under these conditions, one would predict an excess of potential binding sites on the organic ligands relative to the Hg concentration in the water. These considerations suggest that most dissolved Hg in our experimental treatments was complexed with organic ligands.

The speciation behavior of the inorganic fraction is much more straightforward. In oxygenated water of low ionic strength, most dissolved Hg (II) will be in uncharged OH^- and Cl^- complexes (Benes and Havlik, 1979; Gutknecht, 1981). We did not measure Cl^- in our experimental treatments, but in a previous study, Cl^- in water from the source stream ranged from 5 to 50 μM (Newman, 1986). Based on the concentrations of cations we measured, Cl^- concentration in the test water was probably near the lower end of this range. Given a low Cl^- concentration and pH averaging 6.8, the dominant inorganic Hg species under our exposure conditions should be very similar to those reported by Wicklund Glynn et al. (1994) for an artificial soft water; about 90% as $Hg(OH)_2$, about 10% as HgOHCl, and traces of other OH^- and Cl^- complexes.

We did not attempt to fractionate inorganic and organic Hg species in our treatments, but instead analyzed the water for total Hg. When fish were not present in aquaria, Hg concentrations in the test water declined over 24 h, especially at higher initial concentrations. It is important to note that this decline was not due to the added Hg complexing with humic substances present in the water. These complexes were broken down by the digestion procedure before analysis, and Hg bound to organic ligands in solution would be detected as total Hg by this procedure. Instead, the decreases in Hg over time were probably due to sorption of Hg on the inner surfaces of the aquaria.

When fish were present, the rate of Hg disappearance from the test solutions was much more rapid. Fish mucus has considerable affinity for Hg (McKone et al., 1971; Varanasi et al., 1975), so surfaces of fish when present represented additional sorption sites, as would debris such as sloughed mucus or fecal material at the bottom of the aquaria. We did not observe increased mucus production on gills or body surfaces with Hg exposure. Apparently, the amount of mucus normally present was sufficient to trap much of the Hg added daily with the water changes. We sampled water near the surface of the aquaria and avoided particulate debris from the bottom of the tanks to better estimate the fraction of added Hg actually remaining in solution. Any Hg bound to particulate material or sorbed to the walls of the tank was probably not available for uptake at the gills, and would therefore not be expected to produce responses in the gill tissues.

It is also important to note that organically-complexed Hg (II) forms are not equivalent to organic Hg species, such as methyl mercury (MeHg). Methylation involves covalent bonding of Hg to C (in CH_3), whereas organic complexation represents binding or sorption of Hg (regardless of species) to an organic ligand (Benes and Havlik, 1979). In natural waters, methylation of Hg can occur in sediments or in the water column, but even under conditions that enhance methylation, only 2 or 3% of added Hg (II) is converted to MeHg in 24 h (Winfrey and Rudd, 1990). Given the daily replacement of water and renewal of Hg (II), it is unlikely that significant amounts of MeHg were present in our experimental aquaria. Since fish cannot methylate Hg in vivo (Huckabee et al., 1978), the effects we observed must have been due to inorganic Hg species, or organically complexed Hg (II). However, for many metals, organically-complexed species are less bioavailable and less toxic than inorganic species; examples include Al (Witters et al., 1990), Cu (Winner, 1984) Pb (Campbell and Evans, 1987) and Zn (Paulauskis and Winner, 1988). If this is also the case for Hg, then our measurements of total Hg overestimate the amount of biologically-active Hg present. The combination of loss of Hg from water via sorption, and the organic complexation of most dissolved Hg suggest that fish were probably exposed to far lower concentrations of inorganic Hg species than indicated by the nominal treatment values.

Unfortunately, many previous studies of Hg-induced changes in gill morphology do not report the chemical composition of the exposure water, making interpretation and comparison of their results difficult. In the only other study of effects of sublethal Hg exposure on gill morphology we are aware of, Hg was continuously added to filtered seawater for final concentrations of 25 and 50 nM (Periera, 1988). In seawater, the dominant Hg species are negatively charged Cl^- complexes, $HgCl_4^{2-}$ and $HgCl_3^-$ (Benes and Havlik, 1979; Gutknecht, 1981). Periera (1988) also observed changes in the number of chloride cells in Hg-exposed fish, suggesting that ion regulation is impaired by both neutral Hg (II) species, as in the present study, and negatively charged forms.

Lock et al. (1981) observed that plasma Na and Cl concentrations decreased when fish were exposed to both Hg (II) and MeHg. They found that exposure to sublethal Hg concentrations increased gill permeability to water, implying changes in gill ion permeability as well. This increase would lower plasma ion levels by increasing diffusive ion losses and water uptake by osmosis. Under these circumstances, two broad 'strategies' are available to maintain ionic homeostasis. First, the passive ionic efflux could be decreased, perhaps by thickening epithelia to increase diffusion distances. Second, active uptake of ions could be increased to make up for the diffusive losses. Each of these strategies entails costs; the former would increase the diffusive barrier for gas exchange and impair O_2 uptake and CO_2 excretion (Bindon et al., 1994), while the latter requires ATP, thus increasing energy expenditures. The changes in gill morphology that we observed are consistent with the strategy of increased active uptake, but suggest that diffusion distances do not increase to limit passive losses.

We observed an increase in the volume density of chloride cells in all Hg treatments. Chloride cells are the site of active ion uptake in freshwater fish (Laurent et al., 1985; Perry et al., 1992), so the observed increase presumably represents increased

transport capacity. Increased volume density of a cell type in a tissue can result from an increase in cell number (hyperplasia), an increase in cell size (hypertrophy), or some combination of the two. We did not count or measure individual chloride cells, so we cannot quantitatively demonstrate the basis of the increased volume density. From qualitative observations of the sectioned material (Fig. 5), numbers of chloride cells appeared to increase with Hg exposure, and there may have been some increase in average cell size as well. We observed that the number of chloride cells appeared to increase in the primary lamellar epithelium, but not along the secondary lamellae with Hg exposure. Chloride cell numbers increase on secondary lamellae in response to a variety of ionic stressors (Leino and McCormick, 1984; Laurent et al., 1985; Karlsson-Norrgren et al., 1986), so the absence of such an increase here is puzzling. Some metals may accumulate in chloride cells, damaging them and decreasing their numbers over time, especially along secondary lamellae (Oronsaye and Brafield, 1984; Evans et al., 1988). However, Hg does not appear to accumulate in chloride cells, as discussed below.

The primary lamellar epithelium became thicker with Hg exposure. Chloride cells differentiate from stem cells that divide in the primary lamellar epithelium (Conte and Lin, 1967; Chretien and Pisam, 1986; Laurent et al., 1994), so thickening may be associated with increased chloride cell production, or increased chloride cell size. Epithelial thickening also lengthens the diffusion distance between plasma and external water, and would be expected to decrease passive fluxes of ions and water. However, the width of secondary lamellae did not change with Hg exposure. If the epithelium of secondary lamellae became thicker, the width of secondary lamellae should have increased. The secondary lamellae have a large surface area, extensive vascularization and thin epithelia, reflecting their role as the major site of gas exchange. Because of these features, secondary lamellae must represent a major site of passive ion and water flux, and one would expect an increase in the thickness of the epithelium of the secondary lamellae to decrease diffusional losses. The diffusion distance in secondary lamellae increases with exposure to low pH and some metals (Hughes et al., 1979; Karlsson-Norrgren et al., 1985; Tietge et al., 1988), due to cell proliferation, cellular swelling, or an increase in the volume of extracellular fluids. The absence of an increase in the thickness of the secondary lamellar epithelium suggests that blood–water diffusion distances did not increase in response to Hg exposure.

The external surfaces of gill epithelial cells normally have an elaborate system of microridges, which may serve to increase cell surface area, induce microturbulence at the gill surface, or anchor surficial mucus (Hughes and Wright, 1970). These microridges were smaller and less distinct in fish exposed to Hg. Microridge loss also occurs upon exposure to low pH (Jagoe and Haines, 1983) and metals including Cr (Temmink et al., 1983) and Cd (Karlsson-Norrgren et al., 1985). Periera (1988) also observed altered microridge patterns including shortening and fragmentation in flounder chronically exposed to Hg in seawater. The loss of microridges is not due to accumulated mucus obscuring the surface patterns, because routine fixation and processing for SEM removes the mucus coating. Instead, the loss may result from swelling of the pavement cells, expanding the apical surfaces while shrinking and spreading the ridges, much as when a balloon is inflated. Regardless of their exact

function, microridge loss doubtlessly interferes with gill function, and if the interpretation of loss via cellular swelling is correct, suggests alterations in osmotic status of the surficial cells.

Because of their ion-transporting function, chloride cells can accumulate potentially toxic metals when these are present in water (Karlsson-Norrgren et al., 1986; Youson and Neville, 1987; Wicklund Glynn et al., 1994). We stained tissues to detect Hg using an autometallographic technique, and deposition of the metal did not appear to be associated with chloride cells. Wicklund Glynn et al. (1994), using a different technique (autoradiography), reached the same conclusion. Their results showed no specific cell type was associated with accumulation of Hg (II) from water, and the metal appeared evenly distributed through out the epithelium. In contrast, they reported that radiocadmium concentrated in chloride cells, apparently accumulated by ion exchange mechanisms normally transporting Ca^{2+}. Olson and Fromm (1973) were also unable to localize an uptake site for waterborne Hg using an x-ray microanalysis technique. All these studies are consistent with the conclusion that chloride cells do not accumulate Hg at sublethal doses in freshwater. This may be related to the speciation behavior of Hg (II) in freshwater, where uncharged OH^- and Cl^- complexes predominate.

Because chloride cells do not appear to actively take up Hg from water, it is difficult to explain why gill Na^+,K^+-ATPase, mostly localized in chloride cells, is inhibited by Hg. Some studies demonstrating Na^+,K^+-ATPase inhibition involved exposure to acutely lethal, or very high concentrations of Hg (Renfro et al., 1974; Borquegneau, 1977), and Lock et al. (1981) only observed inhibition at lethal Hg concentrations. Under these circumstances, enough Hg probably accumulated in the gill epithelium to interfere with a variety of enzymes, including Na^+,K^+-ATPase, even though the chloride cells were not specifically accumulating the metal. Recently, however, Stagg et al. (1992) reported a correlation between body burden of Hg and gill Na^+,K^+-ATPase activity in flounders collected from an Hg-polluted estuary. Although they did not measure dissolved Hg in water at the collection sites, these fish presumably accumulated Hg via their diet. This suggests that Hg need not enter fish via the gills to produce osmoregulatory effects.

Concentrations of dissolved Hg in natural waters are typically quite low, except in heavily polluted locations (Fitzgerald, 1979). In most cases, dietary Hg is the dominant exposure pathway for biota (Phillips and Buhler, 1978; Dallinger et al., 1987). Wright and Welbourne (1993) demonstrated that dietary Hg affected Na and Ca regulation in crayfish (*Orconectes propinquus*), although they did not measure Na^+,K^+-ATPase activity or examine the morphology of osmoregulatory tissues. Wobeser (1975b) observed gill abnormalities in trout fingerlings fed elevated levels of MeHg. These studies suggest that sublethal levels of Hg, accumulated via realistic pathways, may affect ion and osmoregulation. Our study showed that Hg caused changes in gill morphology consistent with altered ionic fluxes, and that these changes are similar to those produced by other metals known to affect ion regulation. Further studies appear warranted, perhaps correlating gill morphological features with Hg burden and physiological measurements such as plasma ion levels and ATPase activities, and testing effects of Hg via dietary exposure.

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References

Andersen, J.T. and E. Baatrup (1988) Ultrastructural localization of mercury accumulations in the gills, hepatopancreas, midgut and antennal glands of the brown shrimp, *Crangon crangon*. Aquat. Toxicol. 13, 309–324.

American Public Health Association, American Water Works Association and Water Pollution Control Federation (1985) Standard Methods for the Examination of Water and Wastewater, Sixteenth Edition. American Public Health Association, Washington, DC.

Benes, P. and B. Havlik (1979) Speciation of mercury in natural waters. In: The Biogeochemistry of Mercury in the Environment, edited by J.O. Nriagu. Elsevier/North Holland Biomedical Press, Amsterdam, pp. 175–202.

Bindon, S.D., J.C. Fenwick and S.F. Perry (1994) Branchial chloride cell proliferation in the rainbow trout, *Oncorhynchus mykiss*: implications for gas transfer. Can. J. Zool. 72, 1395–1402.

Bolender, R.P. (1979) Morphometric analysis in the assessment of the response of the liver to drugs. Pharmacol. Rev. 30, 429–443.

Booth, C.E., D.G. McDonald, B.P. Simons and C.M. Wood (1988) Effects of aluminum and low pH on net ion fluxes and ion balance in the brook trout (*Salvelinus fontinalis*). Can. J. Fish. Aquat. Sci. 45, 1563–1574.

Borquegneau, J.M. (1977) ATPase activity in mercury intoxicated eels. Experientia 33, 941–942.

Campbell, J.H. and R.D. Evans (1987) Inorganic and organic ligand binding of lead and resultant implications for bioavailability. Sci. Total Environ. 62, 219–277.

Chretien, M. and M. Pisam (1986) Cell renewal and differentiation in the gill epithelium of fresh- or salt-water-adapted euryhaline fish as revealed by (H^3)-thymidine radioautography. Biol. Cell. 56, 137–150.

Conte, F.P. and D.H.Y. Lin (1967) Kinetics of cellular morphogenesis in gill epithelium during seawater adaptation of *Oncorhynchus* (Walbaum). Comp. Biochem. Physiol. 23, 945–957.

Dallinger, R., F. Prosi, H. Segner and H. Back (1987) Contaminated food and uptake of heavy metals by fish: a review and proposal for further research. Oecologia 73, 91–98.

Danscher, G. (1984) Autometallography. A new technique for light and electron microscopic visualization of metals in biological tissue (gold, silver, metal sulphides and metal selenides). Histochemistry 81, 331–335.

Daoust, P.Y., G. Wobeser and J.D. Newstead (1984) Acute pathological effects of inorganic mercury and copper in gills of rainbow trout. Vet. Pathol. 21, 93–101.

Elias, H. and D.M. Hyde (1983) A Guide to Practical Stereology. Karger, New York.

Evans, R.E., S.B. Brown and T.J. Hara (1988) The effects of aluminum and acid on the gill morphology in rainbow trout. Environ. Biol. Fish. 22, 299–311.

Fitzgerald, W.F. (1979) Distribution of mercury in natural waters. In: The Biogeochemistry of Mercury in the Environment, edited by J.O. Nriagu. Elsevier/North Holland Biomedical Press, Amsterdam, pp. 161–173.

Freda, J., D.A. Sanchez and H.L. Bergman (1991) Shortening of branchial tight junctions in acid-exposed rainbow trout (*Oncorhynchus mykiss*). Can. J. Fish. Aquat. Sci. 48, 2028–2033.

Gutknecht, J. (1981) Inorganic mercury (Hg^{2+}) transport through lipid bilayer membranes. J. Membrane Biol. 61, 61–66.

Hinton, D.E. (1993) Toxicologic histopathology of fishes: a systematic approach and overview. In: Pathobiology of Marine and Estuarine Organisms, edited by J.A. Couch and J.W. Fournie. CRC Press, Boca Raton, FL, pp. 177–215.

Hinton, D.E., R.C. Lantz, J.A. Hampton, P.R. McCuskey and R.S. McCuskey (1987) Normal versus abnormal structure: considerations in morphologic responses of teleosts to pollutants. Environ. Health Persp. 71, 139–146.

Huckabee, J.W., S.A. Janzen, B.G. Blaylock, Y. Talmi and J.J. Beauchamp (1978) Methylated mercury in brook trout (*Salvelinus fontinalis*): absence of an in vivo methylating process. Trans. Am. Fish. Soc. 107, 848–852.

Hughes, G.M. and D.E. Wright (1970) A comparative study of the ultrastructure of the water/blood pathways in the secondary lamellae of teleost and elasmobranch fishes. Z. Zellforsch. 104, 478–493.

Hughes, G.M. and S.F. Perry (1976) Morphometric study of trout gills: a light-microscopic method suitable for the evaluation of pollutant action. J. Exp. Biol. 64, 447–460.

Hughes, G.M., S.M. Perry and V.M. Brown (1979) A morphometric study of the effects of nickel, chromium and cadmium on the secondary lamellae of rainbow trout gills. Water Res. 13, 665–679.

Humason, G.L. (1967) Animal Tissue Techniques. W.H. Freeman and Co., San Francisco.

Jagoe, C.H. (1995) Responses at the tissue level: quantitative methods in histopathology applied to ecotoxicology. In: Ecotoxicology, a Hierarchical Treatment. Savannah River Symposia in Environmental Sciences, Volume 2., edited by M.C. Newman and C.H. Jagoe. Lewis Publishers, Boca Raton, FL, in press.

Jagoe, C.H. and T.A. Haines (1983) Alterations in gill epithelial morphology of yearling Sunapee trout exposed to acute acid stress. Trans. Am. Fish. Soc. 112, 689–695.

Jagoe, C.H. and T.A. Haines (1990) Morphometric effects of low pH and limed water on the gills of Atlantic salmon (*Salmo salar*). Can. J. Fish. Aquat. Sci. 47, 2451–2460.

Jagoe, C.H., V.E. Matey, C.A. Haines and V.T. Komov (1993) Effect of beryllium on fish in acid water is analogous to aluminum toxicity. Aquat. Toxicol. 24, 241–256.

Jensen, P.K. and E. Baatrup (1988) Histochemical demonstration of mercury in the intestine, the nephridia and the epidermis of the marine polychaete, *Nereis virens*, exposed to inorganic mercury. Mar. Biol. 97, 533–540.

Karlsson-Norrgren, L., P. Runn, C. Haux and L. Forlin (1985) Cadmium induced changes in gill morphology of zebrafish *Brachydanio rerio* (Hamilton-Buchanan) and rainbow trout *Salmo gairdneri* Richardson. J. Fish. Biol. 27, 81–95.

Karlsson-Norrgren, L., I. Bjorkland, O. Ljungberg and P. Runn (1986) Acid water and aluminum exposure: experimentally induced gill lesions in brown trout *Salmo trutta* L. J. Fish Dis. 9, 11–25.

Khangarot, B.S. and R.C. Somani (1980) Toxic effects of mercury on the gills of a freshwater teleost, *Puntius sophore* Hamilton. Curr. Sci. 49, 832–834.

Larsson, A., B.E. Bengtsson and C. Haux (1981) Disturbed ion balance in flounder, *Platichthys flesus*, exposed to sublethal levels of cadmium. Aquat. Toxicol. 1, 19–35.

Lauren, D.J. and D.G. McDonald (1985) Effect of copper on branchial ionoregulation in the rainbow trout *Salmo gairdneri* Richardson. Modulation by water hardness and pH. J. Comp. Physiol. B 155, 635–644.

Laurent, P., H. Hobe and S. Dunel-Erb (1985) The role of environmental sodium chloride relative to calcium in gill morphology of freshwater salmonid fish. Cell Tiss. Res. 240, 675–692.

Laurent, P., S. Dunel-Erb, C. Chevalier and J. Lignon (1994) Gill epithelial kinetics in a freshwater teleost, *Oncorhynchus mykiss*, during adaptation to ion-poor water and hormonal treatments. Fish Physiol. Biochem. 13, 353–370.

Leino, R.L. and J.H. McCormick (1984) Morphological and morphometrical changes in chloride cells of the gills of *Pimephales promelas* after chronic exposure to acid water. Cell Tiss. Res. 236, 121–128.

Leino, R.L. and J.H. McCormick (1993) Responses of juvenile largemouth bass to different pH and aluminum levels at overwintering temperatures: effects on gill morphology, electrolyte balance, scale calcium, liver glycogen and depot fat. Can. J. Zool. 71, 531–543.

Lock, R.A.C., P.J.M. Cruijsen and A.P. van Overbeeke (1981) Effects of mercuric chloride and methylmercuric chloride on the osmoregulatory function of the gills in rainbow trout, *Salmo gairdneri* Richardson. Comp. Biochem. Physiol. 68C, 151–159.

McDonald, D.G. (1983) The effects of H^+ upon the gills of freshwater fish. Can. J. Zool. 61, 691–703.
McDonald, D.G., J.P. Reader and T.K.R. Dalziel (1989) The combined effects of pH and trace metals on fish ionoregulation. In: Acid Toxicity and Aquatic Animals. Society for Experimental Biology Seminar Series, edited by R. Morris, D.J.A. Brown, E.W. Taylor and J.A. Brown. Cambridge University Press, Cambridge, pp. 221–242.
McKone, C.E., R.G. Young, C.A. Bache and D.J. Lisk (1971) Rapid uptake of mercuric ion by goldfish. Environ. Sci. Technol. 5, 1138–1139.
Newman, M.C. (1986) Comprehensive cooling water report, Volume 2. Water quality. Publication 28-II, University of Georgia, Savannah River Ecology Laboratory, Aiken, SC.
Olson, K.R. and P.O. Fromm (1973) Mercury uptake and ion distribution in gills of rainbow trout (*Salmo gairdneri*): tissue scans with an electron microprobe. J. Fish. Res. Board Can. 30, 1575–1578.
Oronsaye, J.A.O. and A.E. Brafield (1984) The effects of dissolved cadmium on the chloride cells of the gills of the stickleback, *Gasterosteus aculeatus* L. J. Fish Biol. 25, 253–258.
Paulauskis, J.D. and R.W. Winner (1988) Effects of water hardness and humic acid on zinc toxicity to *Daphnia magna* Strauss. Aquat. Toxicol. 12, 273–290.
Paulose, P.V. (1987) Accumulation of organic and inorganic mercury and histological changes in the gills of *Gambusia affinis*. Proc. Indian Nat. Sci. Acad. B 53, 235–237.
Pereira, J.J. (1988) Morphological effects of mercury exposure on windowpane flounder gills as observed by scanning electron microscopy. J. Fish Biol. 33, 571–580.
Perry, S.F., G.G. Goss and P. Laurent (1992) The interrelationships between gill chloride cell morphology and ionic uptake in four freshwater teleosts. Can. J. Zool. 70, 1775–1786.
Phillips, G.R. and D.R. Buhler (1978) The relative contributions of methylmercury from food or water to rainbow trout *Salmo gairdneri* in a controlled laboratory environment. Trans. Am. Fish. Soc. 107, 853–861.
Renfro, J.L., B. Schmidt-Nielsen, D. Miller, D. Benos and J. Allen (1974) Methyl mercury and inorganic mercury: uptake, distribution and effect on osmoregulatory function in fishes. In: Pollution and Physiology of Marine Organisms, edited by F.J. Vernberg and W.B. Vernberg. Academic Press, New York, pp. 101–122.
Sokal, R.R. and F.J. Rohlf (1981) Biometry. W.H. Freeman and Co., San Francisco.
Spry, D.J. and C.M. Wood (1985) Ion flux rates, acid base status and blood gases in rainbow trout, *Salmo gairdneri* exposed to toxic zinc in natural softwater. Can. J. Fish. Aquat. Sci. 42, 1332–1341.
Stagg, R.M., J. Rusin and F. Brown (1992) Na^+,K^+-ATPase activity in the gills of the flounder (*Platichthys flesus*) in relation to mercury contamination in the Firth of Forth. Mar. Environ. Res. 33, 255–266.
Temmink, J.H.M., P.J. Bouwmeister, P. DeLong and J.H.J. Van den Berg (1983) An ultrastructural study of chromate-induced hyperplasia in the gill of a rainbow trout (*Salmo gairdneri*). Aquat. Toxicol. 4, 165–179.
Tietge, J.E., R.D. Johnson and H.L. Bergman (1988) Morphometric changes in gill secondary lamellae of brook trout (*Salvelinus fontinalis*) after long-term exposure to acid and aluminum. Can. J. Fish. Aquat. Sci. 45, 1643–1648.
Varanasi, U., P.A. Robisch and D.C. Malins (1975) Structural alterations in fish epidermal mucus produced by water-borne lead and mercury. Nature 258, 431–433.
Weibel, E.W., G.S. Kistler and W.F. Scherle (1966) Practical methods for morphometric cytology. J. Cell. Biol. 30, 23–38.
Wicklund Glynn, A., L. Norrgren and A. Mussener (1994) Differences in uptake of inorganic mercury and cadmium in the gills of the zebrafish, *Brachydanio rerio*. Aquat. Toxicol. 30, 13–26.
Wilson, R.W., H.L. Bergman and C.M. Wood (1994) Metabolic costs and physiological consequences of acclimation to aluminum in juvenile rainbow trout (*Oncorhynchus mykiss*). 2: Gill morphology, swimming performance and aerobic scope. Can. J. Fish. Aquat. Sci. 51, 536–544.
Winfrey, M.R. and J.W.M. Rudd (1990) Environmental factors affecting the formation of methylmercury in low pH lakes. Env. Tox. Chem. 9, 853–869.
Winner, R.W. (1984) The toxicity and bioaccumulation of cadmium and copper as affected by humic acid. Aquat. Toxicol. 5, 267–274.

Witters, H.E., S. Van Puymbroeck, J.H.D. Vangenechten and O.L.J. Vanderborght (1990) The effect of humic substances on the toxicity of aluminium to adult rainbow trout, *Oncorhynchus mykiss* (Walbaum). J. Fish Biol. 37, 43–53.

Wobeser, G. (1975a) Acute toxicity of methylmercury chloride and mercuric chloride for rainbow trout (*Salmo gairdneri*) fry and fingerlings. J. Fish. Res. Board Can. 32, 2005–2013.

Wobeser, G. (1975b) Prolonged oral administration of methyl mercury chloride to rainbow trout (*Salmo gairdneri*) fingerlings. J. Fish. Res. Board Can. 32, 2015–2023.

Wood, C.M. (1992) Flux measurements as indices of H^+ and metal effects on freshwater fish. Aquat. Toxicol. 22, 239–264.

Wright, D.H. and P.M. Welbourn (1993) Effects of mercury exposure on ionic regulation in the crayfish *Orconectes propinquus*. Environ. Poll. 82, 139–142.

Youson, J.H. and C.M. Neville (1987) Deposition of aluminum in the gill epithelium of rainbow trout (*Salmo gairdneri* Richardson) subjected to sublethal concentrations of the metal. Can. J. Zool. 65, 647–656.